GENE THERAPY FOR DIABETES: LENTIVIRAL EXPRESSION OF INSULIN IN LIVER CELLS

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CERTIFICATE OF AUTHORSHIP

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that this thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Prudence Nicol Gatt

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All I need is a little sign,
To get behind this sun and cast this weight of mine,
All I need is the place to find,
And there I'll celebrate.

~All I Need; Air

PUBLICATIONS AND PRESENTATIONS

Ren B, O'Brien B, Byrne M, Ch'ng E, **Gatt P.N.**, Swan M.A., Nassif N, Wei M.Q., Gigsbers R, Debyser Z and Simpson A.M. Long term reversal of Diabetes in Non-Obese Diabetic mice by liver-directed gene therapy (in preparation).

Ren B, **Gatt P**, Simpson A.M (2009). Correction of diabetes in mice following transplantation of an insulin-secreting human liver cell line. Poster Presentation at IPITA-IXA, October 2009.

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ABBREVIATIONS

α	alpha
β	beta
β2M	beta two microglobulin
δ	delta
γ	gamma
°C	degrees celsius
Ψ	packaging signal
ABS	Australian Bureau of Statistics
AIHW	Australian Institute of Health of Welfare
APC	antigen-presenting cell
$CD4^+$	T-cell co-receptor
$CD8^+$	T-cell co-receptor
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane conductance regulator
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOPE	1, 2-dioleyl-sn-glycro-3-phosphoethanolamine
DOTAP	1, 2-dioleyl-sn-glycro-3-trimethylammonium-propane
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EF1-α	elongation factor 1-alpha
ESRD	end-stage renal disease
FBS	foetal bovine serum
FFO	intervallic infusion in full flow occlusion
h	hour
H_2O	water
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMD	HIV/murine stem cell virus promoter/enhancer hybrid
HPRT	Hypoxanthinephophoribosyltransferase
IDF	International Diabetes Federation
IFN-γ	interferon gamma
IL	interleukin
INS-FUR	furin cleavable human proinsulin
IVGTT	intravenous glucose tolerance test
LTR	long term repeat
M	molarity
MHC	major histocompatibility complex
min	minute
MOI	multiplicity of infection (a ratio of viral infectious particles per cell)
MSCV	murine stem cell virus

$N_2(l)$	liquid nitrogen
NOD	non-obese diabetic
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
O/N	overnight
PCR	polymerase chain reaction
ppm	parts per million
PS	penicillin and streptomycin
pWPT	elongation factor 1-alpha/woodchuck hepatitis virus posttranscriptional regulatory element promoter/enhancer
q	quantitative
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
s	second
SCID	severe combined immunodeficiency
STZ	streptozotocin
T-cell	T-lymphocyte (white blood cell)
TFF	tangential flow filtration
T1D	Type 1 diabetes mellitus
TNF-α	Tumour Necrosis Factor alpha
T_M	melting temperature
U	units
µ	micro
µL	microlitre
v/v	volume/volume
WHO	World Health Organisation
WPRE	woodchuck hepatitis virus posttranscriptional regulatory element
w/v	weight/volume

ABSTRACT

Type 1 diabetes mellitus (T1D) is caused by the autoimmune destruction of the insulin producing pancreatic beta (β)-cells. Current treatment is by multiple daily insulin injections, which cannot mimic the minute-to-minute responsiveness of β-cells *in vivo*, leading to the development of chronic complications, which increase morbidity and mortality. This study investigated the use of lentiviral vectors to deliver the furin-cleavable proinsulin gene (INS-FUR) to liver cells with the goal of generating glucose-responsive, insulin secreting surrogate β-cells.

A methodology for viral titre determination using flow cytometry was developed, optimised and validated. This protocol was compared to the use of fluorescence microscopy for titre determination. The latter underestimated the viral titre as accurate quantification of transduced cells (enhanced green fluorescent protein (EGFP)-positive) was not possible due to the inability to count individual cells, which were in confluent monolayer clusters. Furthermore, cells transduced using a low multiplicity of infection (MOI; a ratio of viral infectious particles per cell) were indistinguishable from background fluorescence, while high MOI significantly underestimated the true functional viral titre. Flow cytometry enabled the determination of single or low MOI events and thus increased the accuracy of the viral titre determination.

The second aim of the study was to examine liver tissue from spontaneous diabetic non-obese diabetic (NOD) mice, which had normalised their blood glucose following the delivery of furin-cleavable insulin in the HIV/murine stem cell virus promoter/enhancer hybrid (HMD) lentiviral vector (HMD/INS-FUR), for evidence of pancreatic transcription factors and hormones, indicative of liver to pancreas transdifferentiation. The HMD/INS-FUR-treated NOD mice normalised blood glucose levels 24 h after viral vector delivery and normoglycaemia was maintained for 150 days (experimental end point). The transduced liver tissue showed the presence of insulin storage granules and the expression of several pancreatic transcription factors and hormones, including *Pdx-1*, *Neurod1*, *Ngn3*, *Pax 4*, *Nkx2.2*, glucagon and somatostatin. Furthermore, the induction of mouse insulin 1

expression was detected and this phenomenon may be attributable to the expression of *MafA* and *MafB* transcription factors, which are known to play crucial roles in insulin expression late in the hierarchy of β-cell maturation. These results indicated that the delivery HMD/INS-FUR lentiviral vector to liver tissue induces hepatocyte to β-cell transdifferentiation and therefore holds therapeutic potential for the reversal of autoimmune T1D.

The final aim of this study investigated lentiviral vector construct design, and compared the HMD/INS-FUR vector construct with the newly acquired WPT/INS-FUR lentiviral construct, which possessed the elongation factor 1-alpha promoter with the woodchuck hepatitis virus post-transcriptional regulatory element enhancer (WPT). The ability of each lentiviral vector to transduce the Huh7 liver cell line using various MOI, insulin secretion, storage and glucose-regulated secretion were compared. Huh7 cells were readilty transduced with the HMD/INS-FUR vector at an MOI of 75, however attempts at transducing the cells with the WPT/INS-FUR vector at an MOI >50, resulted in inhibition of cell growth. This was not the case with the empty-WPT vector, which implies the INS-FUR gene expressed in this vector putatively inhibited metabolic cell function at the higher MOI of 75. Insulin secretion was the same irrespective of the MOI used to transduce the Huh7 cells. By comparison, the WPT/INS-FUR viral vector-transduced cells stored a significantly higher concentration of insulin when compared to cells transduced with the 50 and 75 MOI of the HMD/INS-FUR viral vector ($p < 0.0001$ and $p = 0.001$, respectively). Therefore, fewer viral particles of the WPT/INS-FUR virus were required to achieve similar insulin secretion concentrations as the HMD/INS-FUR virus, while also achieving a higher concentration of insulin storage. However, as the total level of insulin stored after transfection with the WPT/INS-FUR viral vector was low (0.386 ± 0.041 pmoles/10^6 cells), these results must be viewed with caution.

Overall, this study has optimised an appropriate methodology for the accurate determination of viral titre for downstream applications. Furthermore, the study demonstrated the NOD mice transduced with the HMD/INS-FUR viral vector have the ability to store and secrete insulin in a glucose-regulated manner, through the induction of

pancreatic β-cell transcription factors and hormones. Moreover, the WPT/INS-FUR viral vector was found to be comparable to HMD/INS-FUR viral vector with respect to insulin secretion. However, as the former stored significantly more insulin *in vitro*, this suggests that the WPT/INS-FUR viral vector may be a useful construct for future *in vivo* studies investigating the reversal of T1D in NOD mice via hepatocyte transduction.